EHRLICHIA CHAFFEENSIS REPLICATION SITES IN ADULT DROSOPHILA MELANOGASTER

by

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Abstract

Ehrlichia chaffeensis is a Gram-negative, obligatorily intracytoplasmic bacterium and the causative agent of a tick-borne disease, human monocytic ehrlichiosis. In vertebrates, *E. chaffeensis* exhibits tropism for monocytes /macrophages. However, no clear requirements for cell tropism have been defined in ticks. Previously, our group identified two host genes that control *E. chaffeensis* replication *in vivo* in *Drosophila*. We used these two genes, *CG6364* and *separation anxiety* (*san*) to test the hypothesis that *E. chaffeensis* replicates in arthropod hemocytes. Using the *UAS/GAL4* RNAi system, we generated F1 flies (RNAi flies) and confirmed ubiquitous-or tissue-specific reduction in the transcript levels of the targeted genes. When RNAi flies were screened for *Ehrlichia* infections, we found that when either *CG6364* or *san* were specifically suppressed in the hemocytes or in the fat body *E. chaffeensis* failed to replicate or cause infection. Deletion of these genes in the eyes, wings or the salivary glands did not impact fly susceptibility or bacterial replication within these organs. Our data demonstrate that in *Drosophila, E. chaffeensis* replicates within the hemocytes, the insect homolog of mammalian macrophages, and in the fat body, the liver homolog of mammals. This study provides insights about replication sites of *E. chaffeensis* in arthropods.

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Dedication

To my brother, Ashish and sister, Richa. I miss you lots!

Chapter 1 - Literature Review

Drosophila melanogaster

As an experimental model system to study host-pathogen interactions

An understanding of host- pathogen interactions requires a wide range of genetically amenable organisms and tools for forward and reverse genetics. We can use these model systems to understand mechanisms that have been conserved through evolution in lower organisms to help us decipher intricate host-pathogen mechanisms applicable to mammalian systems.

One powerful system which has been exploited is the fruit fly, *Drosophila melanogaster*. In the past decade, the fruit fly has been used to help us understand many aspects of innate immunity (77). *Drosophila* spend their entire life cycle in decaying organic matter. This lifestyle results in contact with an array of micro-organisms; some of them pathogenic. *Drosophila* have developed powerful mechanisms to protect itself from an environment rich with micro-organisms (77).

The *Drosophila* system also fulfils other prerequisites to be considered the foremost experimental model system. The generation time of the fly is short (13). The entire life cycle of *Drosophila* lasts approximately 12-13 days from forming eggs to hatching to an adult fly (13). Moreover, their growth is temperature dependent (13). At 25^{o}C their life cycle is 10 days, at room temperature (21^{o}C -22^{o}C) it is 12-13 days, and at 18^{o}C the generation time is 19 days (13). Rearing and maintenance is simple and inexpensive. Females have a high fertility rate and can

lay about 2000 eggs in a lifetime (13, 49). The *Drosophila* genome has been sequenced and is one of the most fully annotated eukaryotic genomes (8). This provides a vast source of information regarding the molecular makeup of the organisms and allows for functional and comparative genomics. Information about all genes is annotated. Their description expression profile and mutant availability is available at the Genome Annotation Database (http://flybase.net/annot/). The function of human genes can be revealed by studying their parallels in model organisms. Thus, it becomes an appropriate, unparalleled model system to investigate human diseases.

As a model to decipher bacterial virulence factor

Different strategies have been used to infect flies. For example pricking the insect body with a sharp needle dipped in a bacteria culture can cause disease and even death (17, 77). Another more refined method is microinjection of a precise dose volume allows for carefully controlled infections (78). Both these methods allow activation of microbe-specific immune responses which can be monitored (17). Other methods of infection include feeding flies with bacteria mixed in food or spraying fungal spores onto the exoskeleton (17, 78). These methods resemble the natural environment and natural infection routes. However, only few microbes are known to elicit an immune response in this way. One such bacterial species that induce *Drosophila* immune responses in the absence of physical injury is *Erwinia carotovara* (17).

Several groups have used *Drosophila* as a surrogate model system to study the complex interplay between host and pathogen (17, 25, 35, 40, 83, 86, 95, 121, 129). Fruit flies have been

successfully used to identify several virulence factors (35, 40, 72). For example *Pseudomonas aeruginosa* is highly pathogenic to fruit flies. *Drosophila* use the *pil-chp* transduction system which potentially encodes proteins involved in signal transduction pathway to regulate expression of virulence factors important to controlling infection (35). These genes are known to be also required for twitching and motility (35). Studies have also suggested that that the *pil-chp* transduction system is likely to be important for virulence in mammals (66). *P. aeruginosa* virulence determinants important for mammalian pathogenesis were also cause fly death (30, 52). Flies were also instrumental in helping identify a Rho gap Gtpase toxin which, when injected by the type III secretion system, impaired hemocyte phagocytosis (14). Since, hemocytes share functional similarities with human leukocytes, flies can help in the discovery of mammalian homologs (14). *D. melanogaster* were also used to identify virulence factors of *Serratia marcesens.* Thoracic injections of 50-100 CFU of the DB11 strain caused 100% death within 24h at 20^{o}C (72). Three bacterial mutants with decreased pathogenicity, were also identified (72). In fact, one mutant was also markedly attenuated in a murine lung infection model demonstrating processes in flies may also be applicable to vertebrate models (72). *Drosophila* have also helped in identifying virulence factors of *Mycobacteria*. *Mycobacterium muranium* causes tuberculosis in fish and frogs (32, 112). In flies, it caused infections that resembled *M.tuberculosis* infections in humans (40).

M. muranium infections in adult flies were lethal within eight days and killing of flies is accompanied by widespread tissue damage (40). *M. muranium* accumulated in plasmocytes and did not co-localize with acidified organelles or internalized, dead *E. coli* suggesting successful macrophage subversion by *M. Muranium* (40). A *mag*24 deficient mutant strain of the bacteria,

was less virulent (40). The ability of *M. muranium* to reside in non acidified vacules has also been shown in vertebrate macrophages (15). This demonstrates that fly hemocytes are comparable to macrophages and that fruit flies provide insights to the understanding of human pathogens. Altogether, these studies exemplify the use of *Drosophila* as a useful and a tractable model system to study bacteria-host pathogen relationships and cellular interactions in infected cells.

RNA interference in *Drosophila* and mechanism of gene silencing by dsRNA

In 1998 Fire and Mello discovered that dsRNA triggered a reduction in protein synthesis in *Caenorhabditis elegans* (53). Clemens *et al.* reported a similar phenomenon in *Drosophila* cells (33). Since then, RNA interference (RNAi) has revolutionized and accelerated our knowledge of the role of many genes. RNAi methods allow straight forward 'gene function' or 'loss of function' studies. The RNA-dependent gene silencing process (RNAi) is initiated by short double-stranded (dsRNA) molecules (53). When these dsRNA molecules are introduced ectopically into cells or are endogenously generated, they are processed by Dicer, a type III RNase endonuclease (19). The enzyme mediates dsRNA cleavage and produces small fragments of 21-13 nucleotides in length called small interfering RNA (siRNA's) (19). These siRNA's are loaded into the RNA-induced silencing complex (RISC) which is the effector in the gene silencing process (89). The siRNA duplex unwinds and couples to RISC. The single stranded siRNA in RISC guides the sequence specific binding to the target mRNA's which is then cleaved leading to its degradation (89).

RNAi screens in *Drosophila* cells

Our knowledge of host-pathogen interaction is limited for obligate intracellular bacteria. In particular, host factors needed for replication are not very well known. This knowledge gap is largely due to our inability to manipulate mammalian cells effectively and because of the lack of genetic tools. However, as outlined above, *Drosophila* is used as a model system to address host-pathogen interactions. *Drosophila* offers several advantages for these studies. *Drosophila* cell lines are available and grow at atmospheric CO_2 concentrations (48, 91). The two most commonly used *Drosophila* cell lines used are Schneider line 2 (S2) and Kc 2 (91). The S2 and Kc 2 cell lines were derived from primary cultures of *D. melanogaster* embryonic cells that spontaneously immortalized *in vivo* (49, 122). Interestingly, these cells had hemocyte-like properties such as the ability to adhere to foreign surfaces and to phagocytize a variety of targets (113). Moreover, S2 cells also possess functional toll and immunodeficiency pathways that regulate inflammatory responses such as inducible antimicrobial peptide expression (125). This allows for the manipulation of transduction cascades which share striking similarities to the mammalian immune response. These hemocytes-like cells provide insect equivalents to the mammalian macrophages and allowed the study of pathogens that infect macrophages (18). Additionally, long dsRNA's are easily taken up by *Drosophila* cells and a bathing protocol can be used without the use of any transfection reagent (33). Therefore, it is easy to knock-down a gene of interest in these cells allowing loss of function studies (33). Recently, the availability of genome-wide dsRNA library at the *Drosophila* RNAi screening center (DRSC) added another advantage to the utility of the system, facilitating a high throughput platform to analyze gene functions systematically (23, 56).

Several RNAi screens using *Drosophila* cells have been used to study host-pathogen interactions (9, 10, 29, 38, 41, 105-108, 113, 124). In such screens, hemocyte-like S2 or Kc cells were grown in 96-or 384-well formats and were treated with a library or sub library of double stranded RNA's that carry out highly specific RNAi mediated knockdown of each of the *Drosophila* mRNA's (111). These cells were then assayed for bacterial entry, survival or replication using different read outs such as such as fluorescence, cell level imaging, sub-cellular imaging or luminescence (111). These screens have identified distinct host genes that are essential in establishing the infection by various pathogens with overlapping or redundant specificities. The results from these screens have helped in identifying many different classes of entry receptors that are involved in the uptake of broad range of bacteria. These receptors included PGRP's (113), SR-C1(113), Eater (69), Nimrod (71) and the class B scavenger receptor Peste (9). Some screens have also revealed intracellular components and other downstream process in a variety of pathogens. These include genes required for actin remodeling (Arp 2/3 complex), vesicular transport genes such as COPI and COP II complex, genes required for vesicular trafficking between ER and golgi and genes required for docking the plasma membrane for exocytosis (9, 29, 71, 113, 124). Altogether, these observations suggest that each microbe subverts a unique cellular compartment to complete its replication. For example, *Legionella pneumophila* prevent phagosome lysosome fusion and require the ESCRT (endosomal sorting complex required for transport) machinery to alter the phagosome environment. *Francisella tularensis* escape from phagosomes requires a type III P14 kinase, PI4CA, a ubiquitin-specific peptidase, USP22 and E3 ubiquitin ligase, CDC27 to replicate within the cytoplasm (10, 20, 120).

Limitations and future prospects of cell based RNAi screens in *Drosophila*

In the past few years, RNAi screens in *Drosophila* cells provided insights about bacterial intracellular replication. After candidate genes are identified in *Drosophila*, the next step is the screening of homologs in human cell lines to provide a better understanding of mammalian systems. Additional gene validations can also be done in adult flies using a genome-wide library of transgenic flies (39). The library consist of upstream activating sequence (UAS)-driven inverted repeats specific for most genes which cause gene inactivation *in vivo* (39). Although, RNAi screens allow us to test the function of all genes in a given genome in a rapid, systematic and unbiased manner, it is also important to highlight that "off target effects" can occur and are the major drawback of the technique (22, 70). Off target effects cause degradation of non specific mRNA and subsequently leads to false positive reads (70). However, improving RNAi libraries and revalidation of candidate genes is the best solution to eliminate false positive reads.

In vivo RNAi in *Drosophila*

As a complement to cell-based RNAi screening methods, it is also possible to silence host genes required to combat infection in intact flies. This technology gives us more information about the complex biology of the whole organism. RNAi is cell autonomous in *D. melanogaster* (118, 128) unlike *C. elegans* where RNAi-induced gene silencing is systemic (97). Although this property makes it more difficult to silence genes, it does allow for tissue-specific gene silencing. This spatial and temporal control of gene expression by UAS/ GAL4 system has only been possible after the landmark discovery by Brand and Perrimon (24).

The *Gal 4* gene encodes for a yeast, *Saccharomyces cerevisiae,* transcriptional activation protein which is 881 amino acids long. It controls the gene required for galactose metabolism. The upstream activating sequence (UAS) is the promoter where GAL4 binds (63, 73, 74). Since eukaryotic transcription machinery is highly conserved, this system can also be used in other species. Expression of GAL4 activates reporter genes in *Drosophila* without any deleterious effects (43, 54). Specific gene transcription is done by crossing transgenic flies that carry genes under the control of UAS with transgenic flies that make GAL4 (24).

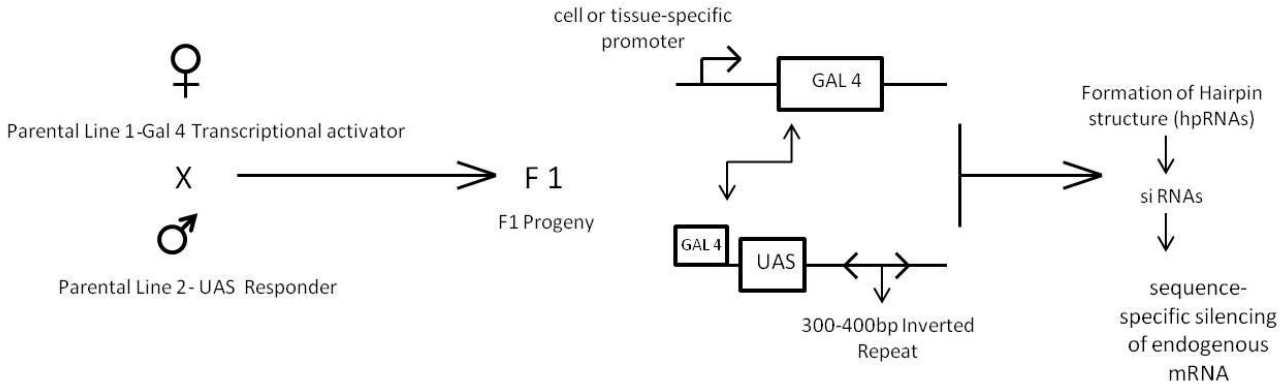

Figure 1.1 Mechanism of transgenic RNAi in *Drosophila* using the GAL4/UAS expression system. Adapted from (7)

The mechanism relies on the GAL4/UAS system to control the expression of a transgene cloned into a vector as inverted repeats. In the progeny, the transgene produces a double-stranded RNA (dsRNA) that forms a hairpin structure, which then triggers a RNAi response (Figure 1.1) (88). The dsRNA is cleaved by the ribonuclease enzyme Dicer (67). *Drosophila* has two paralogs of Dicer, *dcr-1* and *dcr-2* (67). Cleavage of dsRNA by Dicer leads to formation of 21- to 23-nt siRNA (67). The siRNA is then loaded into the multiprotein RISC

complex where the siRNA duplex unwinds (67). Active RISC complex loaded with guide strand then leads to sequence specific degradation of target mRNA (67). This process allows for high specificity in gene targeting.

These RNAi lines were constructed by cloning short gene fragments (300-400bp) as inverted repeats (IRs). First generation vectors used constructs inserted into the genome at random sites using P element transformation (99, 104) . However, this technique had positional effects and generated false negatives (98). This problem was solved by the second generation vector which was called the VALIUM series of vectors. To achieve high integration specificity, a phage, PhiC31, targeted integration method was used (62, 104). This technique permitted integration at sites in the genome that showed optimal expression and optimal RNAi effects were observed (104). These RNAi lines are available from the Vienna Drosophila RNAi collection (VDRC) (39). This library has 15,072 RNAi fly lines targeting 13,327 genes (39, 96). The Transgenic RNAi project (TRIP) collection targets 3,024 genes available (96, 98). These RNAi fly lines, allow genome wide RNAi screens to systematically analyze gene functions in the intact fly. This method also reduces "off target effects" which is a major problem with cell based RNAi screens (104). Moreover, with more transgenic fly lines availability, it is possible to confirm data with a second independent transgenic line.

Recently, a genome-wide *in vivo* RNAi screen identified several *Drosophil*a genes that conferred resistance to the Gram-negative bacterium, *Serratia marcescens* (34). Cronin et al. used 13,053 RNAi transgenic fly lines. This represented 78% of *Drosophila* genome (34). Their initial screen identified susceptible and resistant candidates using ubiquitous (whole organism

expression) RNAi gene silencing. This was followed by the two cell type specific screens where genes were specifically silenced in the gut epithelium and in hemocytes (34). They identified genes involved in intracellular processes, stress response endocytosis, and exocytosis as essential for the bacteria replication in the gut. Genes linked to phagocytosis, vesicle trafficking and stress responses were required in the hemocytes for infection. The JAK-STAT pathway was also identified as an essential host defense process in the gut (34)

In vivo, RNAi screening has not only contributed to a better understanding of host-pathogen interaction, but has also facilitated a better understanding of other relevant fields of biology such as notch signaling (34, 93, 119) heart function (94), muscle development (123) and obesity (109). Therefore, *in vivo* and *in vitro* RNAi screens in *Drosophila* have provided powerful means for biologists to characterize gene functions.

Ehrlichia chaffeensis

Classification

Ehrlichia chaffeensis is classified under the phylum: proteobacteria, order: *Rickettsiales* and family: *Anaplasmataceae* (46, 58, 114). *Rickettsiaceae* and *Anaplasmataceae* are two families in the order *Rickettsiales* (46). Under the family *Anaplasmataceae, f*our genera have been classified namely, *Anaplasama, Ehrlichia, Wolbachia and Neorickettsia* (46). Four organisms have been confirmed to be human pathogens in the family *Anaplasmataceae*: *Ehrlichia chaffeensis, Anaplasma phagocytophilum, Neoricketttsia sennetsu* and *Ehrlichia ewingii* (42). These bacteria are the causative agents of human monocytic ehrlichosis (HME) (11)), human granulocytic anaplasmosis (28), grandular fever (26) and granulocytic ehrlichosis (90) respectively. Recently, a new Ehrlichia species, *Ehrlichia Wisconsin* a close relative of *E.*

muris (98% sequence homology), was reported (110). However, because there are only a few isolates and there is limited genetic data, the exact taxonomic placement has not been determined (110).

Human Monocytic Ehrlichosis and Transmission of the disease

Ehrlichosis is a tick-borne zoonotic disease caused by a Gram-negative, obligatory intracytoplasmic bacterium, *E chaffeensis*. The first case of human monocytic Ehrlichosis was reported in 1986 in a man bitten by ticks in northern Arkansas (50, 102). The bacterium lacks capsules or pili and bind to host cells via the outer membrane (115). After 7- 10 days of infection, inclusions called morula are seen in the cytoplasm of mononuclear cells (117). *E. chaffeensis* is primarily vectored by *Amblyomma americanum* (lone star tick) (12). An important natural reservoir of *E. chaffeensis* in the USA is white tail deer (*Odocoileus virginianus*) (37, 81). This organism is transmitted transtadially in ticks (103). Several other vertebrates may also act as reservoirs including domestic dog (133), domestic goat (45), white footed mouse (85), red fox (36), raccoon (44) and coyote (68). The bacterium enters vertebrate blood via a bite from an infected tick. *A. americanum* has a three-host life cycle and to transform from one stage to the other (larvae – nymph– adult) the molting process in ticks requires a vertebrate blood meal (103). Thus, humans become hosts when ticks bite them accidently.

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Clinical Symptoms

Although HME is one of the diseases reported by the U.S. Centers for Disease Control and Prevention (CDC), it is probably under reported because of its common symptoms. Within 1-2 weeks infected persons show symptoms of malaise, back pain or gastrointestinal symptoms (51, 55). Early symptoms are "flu" like (46). During the course of infection, other symptoms observed are lymphadenopathy, diarrhea, vomiting and abdominal pain (51, 55, 57). About 30-40 % of patients develop a rash in the later course of disease and severe illness is more frequent in immunocompromised individuals (101, 102). Untreated cases may progress to death which may occur as early as the second week of illness (87). Therefore, the disease manifests as a moderate-to-severe disease. Tetracycline/doxycycline is used as a choice of drug. Most patients become afebrile within 1 to 3 days following treatment (46, 101). These drugs reversibly bind to the 30s ribosomal subunit and prevent the formation of peptide chains and thus inhibit bacterial protein synthesis. There is little or no evidence for alternative antibiotic treatments as *in vitro* β lactams, cephalosporins, macrolides and aminoglycosides are inactive against *E.chaffeensis* (126).

Geographic Distribution

The prevalence of ehrlichiosis largely depends on the distribution of tick vectors and reservoir mammalian works (101). *A. americanum* is prevalent in midwestern, south-central and southeastern United States (47). Most cases that have been reported to the U.S. Centers for disease control and prevention (CDC) are from the States of Missouri, Oklahoma, Tennessee, Arkansas and Maryland (100, 126). Ehrlichiosis occurs worldwide and has also been recently reported in China (130), Korea (64), Mali (127) and Peru (31, 84, 92, 127, 130) .

Host Response to *E. chaffeensis* infection and immunology

E. chaffeensis is unique in its strategy to subvert host immune responses. Our knowledge of immune mechanisms following *E. chaffeensis* infection in humans is based mainly on work in rodents. Various inbred mouse strains have been used to study the role of cellular and humoral immunity (59, 61, 131). Wild-type mouse strains clear the bacterium within 16 days while infection persists for one to several months in mice that are defective in macrophage and T-cell functions (61). Mice lacking functional toll-like receptors 4 (*tlr4*) alleles delay bacterial clearance for approximately two weeks as compared to wild-type mice (61). Tlr 4 mutant mice produce decreased levels of nitric oxide and interlukin-6 (IL-6) demonstrating the importance of macrophage stimulation in the clearance of *E chaffeensis* (60). Although, *E. chaffeensis* lack the ability to synthesize LPS, it may be possible that other molecular patters found on *E. chaffeensis* interact with Tlr4 (21, 27). In contrast, *Tlr4* mutant mice appear normal when infected with *A.phagocytophilum* (21). However, blood analysis and necropsy revealed clinical signs such as a neutropenia, monocytosis, and intracytoplasmic morulae in neutrophils for as long as 20 days (21). This suggests that that lack of Tlr4 function may affect the mice longer than has been appreciated (21, 27). Mice lacking functional major histocompatibility complex class II (MHCII) genes are unable to clear *E. chaffeensis* and showed persistent infection suggesting that functional MHCII molecules are essential for clearance of *E. chaffeensis* (61). The role of $CD4^+$T cells and $CD8^+$T cells in the pathogenesis of *E. chaffeensis* has also been investigated (65). The $CD4^+$ T-cell-deficient mice showed increased susceptibility to both high and low doses of *E. chaffeensis* while the $CD8^+$ T-cell-deficient mice were resistant to a low dose but were susceptible with a high dose of *E. chaffeensis* (65). The absence of $CD8^+$ T cells leads to

decrease in TNF-α and IL-10 production, which may be the cause of reduced tissue injury and bacterial burden (65). Based on these observations it is hypothesized that CD8+T cells mediate the disregulated overproduction of TNFα in fatal ehrlichosis, to induce toxic shock (65). In contrast, combined immune deficient mice (SCID), that lack both B and T-cell function, develop severe fatal disease and become moribund 24 days post infection suggesting that both T and B cells are necessary for adaptive immunity during *E. chaffeensis* infection. (131). Mice deficient for T cells but with intact B cell function become infected but do not become ill (131). Moreover, administration of antibodies targeted against the outer membrane protein of *E. chaffeensis* or immune serum from immunocompetent mice in SCID mice result in protection from disease demonstrating a protective role of antibodies which recognize outer membrane proteins of *E. chaffeensis* and the importance of intact cellular immunity for complete recovery (60, 61, 132)

The clinical manifestation of HME is also mediated by other immunological responses such as secretion of variety of cytokines. The cytokine profile in *in vitro* studies using human monocytic cell line (THP-1) that is infected with *E. chaffensis* showed induction of IL-1β, IL-8 and IL-10 while there is no induction of GMCSF, IL-6 and TNFα (75). The absence of TNFα and IL-6 is unique among known intracellular bacteria which suggests that *E. chaffeensis* may need more stringent inhibition of macrophage activation to survive (75). However, additional proinflammatory cytokines such as IL-6 and TNFα are induced when infected cells were exposed to hyperimmune serum containing anti *E chaffeensis* IgG antibodies (76). This suggests that antibodies against *E.chaffeensis* can induce the production of proinflammatory cytokine which may play important role in pathophysiology of the disease and may be detrimental to the host

(76). Another study categorized changes in gene transcription during *E. chaffeensis* infection in THP-1 cells (134). Post infection, a wide range of genes across the host genome are altered. After exposure to *E. chaffeensis* at different time points, transcription of genes involved in biosynthesis and metabolism, ion channel transport, signal transduction, cell differentiation and membrane trafficking are altered three fold (134). The authors focused on the genes that are down regulated which are innate immune response, apoptosis and phagosome lysosome fusion genes (134). In particular, *E. chaffeensis* repressed host-cell cytokine genes: IL-15 and IL-18 that are known to modulate innate and adaptive immunity to intracellular bacteria. These genes are hypothesized as host genes that *E. chaffeensis* down regulate for its survival (134).

Known host factors required for Ehrlichial survival and replication

Our knowledge about host factors that bacteria utilize during entry, survival and replication is nascent. However, some studies have been initiated (16, 79, 80, 116, 134). For example, cholesterol is needed for *Ehrlichia* infection. *E.chaffeensis* that have been treated with Methyl β cyclodextrin, a cholesterol sequesteration and extraction reagent are significantly less invasive (79). It is believed that the cholesterol substitutes for the peptidyl glycan which is not present in the bacterial membrane because *E. chaffeensis* lacks the genes for biosynthesis of peptidoglycan (79). Like many bacteria, iron is critical for *E. chaffeensis* replication (16). Monocytes treated with iron chelators such as deferoxamine, fail to support bacterial replication suggesting the dependence of *E. chaffeensis* on cytoplasmic iron pool (16). Transferrin may be used to scavange iron during replication because exogenously added transferrin is accumulated by *E. chaffeensis* during replication. This suggests that transferrin may be used to scavange iron

during replication (16). Although, aforesaid mentioned studies have found some of the critical host factors *E. chaffeensis* require during replication, other host genes utilized by the bacteria for its survival and replication are yet to be elucidated.

Recently, our group attempted to identify *Drosophila* host genes that are required for *E. chaffeensis* infection (82). The investigators used microarray and mutant screening techniques to identify several host genes that contribute to *E. chaffeensis* replication (82). Experiments in mutant flies confirmed five genes that affect the replication of *E. chaffeensis in vivo.* The functions of the identified genes are diverse and include: phagocytosis/engulfment (*CG6479)* (2); fatty acid binding/mitotic sister chromatid binding (*san)* (5); chitinase activity (*CG3044 (Cht11)*) (4); uridine/cytidine kinase activity (*CG6364*) (1); and enoyl –CoA hydratase activity (*CG6543*) (3). Further analysis of gene products of *CG6364* suggests that *E. chaffeensis* requires cytosine or cytidine for its replication (82). Supplementation of cytosine to S2 cells culture enhanced *Ehrlichia* replication (82). One hypothesis suggested by the investigators is that since cytidine is the least abundant nucleoside in cells, its use by the *Ehrlichia* may be stressful on the host cell processes including host cell defense mechanisms. This would make conditions amenable for *E. chaffeensis* growth (82).

Significance and Conclusion

Ehrlichosis is an emerging infectious disease (42). From 2003-2009 a total of 4,352 cases were reported by CDC with the highest number of cases (944) reported in 2009 (6, 126). Most cases of HME are seen in adults and elderly and since the population of United States has

become older, the growth of a susceptible human population is another factor contributing to emergence (42). Case fatality rate of HME is 3% with deaths occurring most commonly in immunosuppressed individuals (126). The disease has been reported globally and yet there is no vaccine against this emerging infectious disease (31, 90, 130). The complete understanding of the disease's pathogenesis requires the understanding of the genetic requirement and the intracellular niches within which this bacterium replicates. Therefore, in this current work, *Drosophila* RNAi lines were selected from a pilot microarray screen completed earlier in our laboratory (bioinformatics.kumc.edu/mdms/login.php). My goal was to identify tissues in which the bacterium replicates. In this study, I used the UAS/ GAL4 system to control the expression of the target genes in adult flies. Using this system, I tested if we can knock-down a gene of interest in a tissues-specific manner and monitored *E. chaffeensis* infection in adult flies. The study provides valuable information about the intracellular niches where the bacterium replicates.

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Chapter 2 - *Ehrlichia chaffeensis* replication in adult *Drosophila melanogaster*

Abstract

Ehrlichia chaffeensis is a Gram-negative, obligatorily intracytoplasmic bacterium and the causative agent of a tick-borne disease, human monocytic ehrlichiosis. In vertebrates, *E. chaffeensis* exhibits tropism for monocytes /macrophages. However, no clear requirements for cell tropism have been defined in ticks. Previously, our group identified two host genes that control *E. chaffeensis* replication *in vivo* in *Drosophila*. We used these two genes, *CG6364* and *separation anxiety* (*san*) to test the hypothesis that *E. chaffeensis* replicates in arthropod hemocytes. Using the *UAS/GAL4* RNAi system, we generated F1 flies (RNAi flies) and confirmed ubiquitous-or tissue-specific reduction in the transcript levels of the targeted genes. When RNAi flies were screened for *Ehrlichia* infections, we found that when either *CG6364* or *san* were specifically suppressed in the hemocytes or in the fat body *E. chaffeensis* failed to replicate or cause infection. Deletion of these genes in the eyes, wings or the salivary glands did not impact fly susceptibility or bacterial replication within these organs. Our data demonstrate that in *Drosophila, E. chaffeensis* replicates within the hemocytes, the insect homolog of mammalian macrophages, and in the fat body, the liver homolog of mammals. This study provides insights about replication sites of *E. chaffeensis* in arthropods.

Introduction

E. chaffeensis is a an obligate, intracellular bacterium and is the causative agent of human monocytic ehrlichiosis (HME) (4). The disease is usually more severe or fatal in immunosuppressed individuals (14). HME has been reported globally and yet there is no vaccine against this emerging infectious disease (23, 25, 36, 39, 40). *E. chaffeensis* is transmitted primarily by *Amblyomma americanum* (lone star) ticks. In vertebrates, it has tropism for monocytes/macrophages (26). However, the tissue tropism of *E. chaffeensis* in ticks has remained obscure.

To determine where *E. chaffeensis* replicates in arthropods, we used *D. melanogaster*. *Drosophila* have been successfully used to study a wide variety of host-pathogen interactions including the intracellular pathogens *Listeria monocytogenes* (24) , *Mycobacterium marinum* (12) , *Francisella tularensis* (38) and *Plasmodium gallinaceum* (32). Previously, our group demonstrated that *E. chaffeensis* is capable of infecting and completing its life cycle and maintaining infection in both S2 cells and adult flies (21, 22). Two genes, *CG6364* (1) and *san* (2) , were found to control bacterial replication as judged from a microarray screen analysis and follow-up challenge experiments with mutant flies (20). We used tissue-specific gene knock-out techniques to investigate the tissue tropism of *E. chaffeensis.* Specifically, *CG6364* and *san* were silenced using the *GAL 4* (15) transcription factor and the *UAS* promoter (10). This tool allowed us to inactivate the genes of interest ubiquitously or in specific tissues in adult flies to screen for bacterial replication. The working hypothesis is that in *Drosophila, E. chaffeensis* replicate in hemocytes, the insect equivalent of macrophages. Results pointed in the thesis will support the working hypothesis that *Ehrlichia* replicate within the hemocytes and the fat body of adult *D. melanogaster*.

Materials and methods

Cell lines and *E. chaffeensis* infections.

E. chaffeensis (Arkansas isolate) was propagated in DH82 cells (ATTC # CRL-10389, American Type Culture collection, Rockville, Md.), a dog macrophage cell line. The DH82 cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 3.5% fetal bovine serum, 3.5 % Nu serum and 2 mM L-glutamine. Cells were grown at 37°C in 8% CO_2 – 92% air atmosphere. Infectivity was determined by examining cyto-centrifuged cells stained with Dif-Quik stain (Fisher Scientific Company, Kalamazoo, MI; # 122-929). When more than 80% of cells were infected, the cells were removed with a cell scraper and frozen at -80°C in cryogenic vials. Bacteria numbers were quantified using a TaqMan-based quantitative reverse transcriptase PCR (qRT-PCR) assay as described below. Purification of host cell-free bacteria was carried out as follows. Cells were scraped from tissue culture dishes. The recovered infected cells were placed in a 50-ml, sterile centrifuge tube and shaken with glass beads. The preparation was centrifuged at 600 x g for 20 minutes. The supernatant with host cell-free bacteria was transferred into a sterile tube and centrifuged at 15,000 x g for 20 minutes. The bacterial pellet was resuspended in sterile phosphate-buffered saline (PBS) mixed with blue food dye at a ratio of 0.6 ml dye for every 1 ml of PBS to help us visualize appropriate fly injection. Most flies were injected with 6,000 bacteria per fly; however, some early experiments were done with 1,500 bacteria per fly.

D. melanogaster

D. melanogaster flies were raised on standard dextrose/molasses/yeast medium at 18-29°C. The following fly lines were used: *yellow white* (*yw*) and *arm-Gal4, hml ΔGal4, YP1-*

Gal4, MS1096, Gmr-Gal4, Fhk-gal4, UAS-6364 and UAS-San. The *yw* fly line was maintained at Kansas State University. The *arm-Gal,* fly line (31) was received as a gift from Joan Hooper (University of Colorado, Anchutz Medical campus, Denver CO). The *Fhk-gal4* and *YP1-Gal4* was received as a gift from Tony Ip (The University of Massachusetts Medical School, Worcester MA). The *Hml* Δ*Gal4* fly line was received as a gift from Michael Galko (MD Anderson Cancer Center, Houston TX). The *MS1096* and *Gmr-Gal4* fly lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, Bloomington, IN. UAS-dsRNA transgenic lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC) (10).

5-7 virgin females carrying the *Gal4* promoter were crossed with 3-4 males carrying different UAS-dsRNA transgenes to generate F1 flies that had tissue-specific or ubiquitous deletion of *CG6364* and *san* genes.

RNA extraction.

The TriReagent (Molecular Research Center) RNA extraction method, as was previously described by our group (22), was used to extract RNA from flies or host cell-free bacteria. Bacterial pellets or fly homogenates were resuspended in 1 ml of TriReagent. Preparations were transferred to 2.0 ml, Heavy Phase Lock Gel tubes (5 Prime/Eppendorf, Westbury, New York; #2302830). Three hundred microliters (300 µl) of chloroform was added and the mixture was vortexed for 15 seconds. The samples were centrifuged at 12,000 x g for 10 minutes at 4°C and the aqueous phase was transferred to clean 1.5 ml tubes. Five hundred microliters (500 µl) of isopropanol was added and RNA was precipitated at -20°C for 24 hours. Samples were subsequently centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA pellet was washed with 1 ml of 70% ethanol and samples were centrifuged at 7.400 x g for 5 minutes at 4°C. The 70% ethanol was decanted from the pellet and residual ethanol was allowed to evaporate for 5

minutes. RNA was resuspended in 50 µl of nuclease-free water. RNA concentrations were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

Quantification of bacterial numbers for infection/injection and in infected flies.

RNA from host cell-free *Ehrlichia* was extracted as described above. A TaqMan-based real-time reverse transcriptase PCR (RT-PCR) was used to quantify bacterial numbers (34). One hundred fifty-one thousand nano gram of RNA were used for each reaction. Real time quantitative RT-PCR (qRTPCR) was performed using the Invitrogen's One-Step Platinum qRT-PCR kit (#11732) in a Cepheid Smart Cycler (Cepheid,Sunnyvale,CA). *E .chaffeensis* RNA was detected using primers specific for the 16S ribosomal RNA gene (NCBI Accession # M73222). Custom synthesized primers and probes were obtained from Integrated DNA Technologies (Coralville, IA) and were used. The sequence of the primers used were; forward primer, RRG3 (5' CAATTGCTTATAACCTTTTGGTTATAAAT 3') and reverse primer, RRG27 (5' GTATTACCGCGGCTGCTGGCAC 3'). Serial 10-fold dilutions of the RNA from infected DH82 cells were used to generate standard curves plotting log number of bacteria versus the corresponding Ct value. The cycling condition used for the assay were: 48°C for 30 minutes, 94°C for 4 minutes, then 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute.

To quantify bacterial numbers in injected/infected flies, anesthetized flies were transferred to 1.5 ml tubes (Kimble Kontes #749510-1500), and crushed with disposable pestles in 1 ml of TriReagent as previously described (20). Bacteria numbers were estimated from RNA samples using quantitative RT-PCR (qRT-PCR) as described above. *Drosophila* ribosomal *protein 15a* (NCBI Accession #NM_136772) gene was used as used as housekeeping. The sequence of the primers used were: forward primer (5' TGGACCACGAGGAGGCTAGG 3')

and reverse primer (5’ GTTGGTGCATGGTCGGTGA 3’) and Taqman probe (TGGGAGGCAAAATTCTCGGCTTC). The cycling condition used for the assay were: 48°C for 45 minutes, 94°C for 2 minutes, and then 35 cycles of 94°C for 45 seconds, 50°C for 1 minute, and 72°C for 1.5 minutes.

Estimation of silencing efficiency.

To quantify the level of transcriptional silencing in RNAi flies, whole-flies or specific fly tissues were used to probe for specific transcript levels. Fly-tissues were transferred to 1.5 ml tubes and crushed with disposable pestles in 1 ml of TriReagent for RNA extraction. Transcript levels of the gene of interest in F1 flies, were assessed in the following whole-flies: ubiquitous (UAS San X *arm-Gal4* or *UAS-CG6364* X *arm-gal4*); hemocytes (UAS San X *hml-Gal4* or *UAS-CG6364* X *hml-gal4*); fat body (*UAS-San* X *YP1-Gal4* or *UAS-CG6364* X *YP1-Gal4*). For wing-specific and eye-specific knock-down, fly heads or wings were used. For body-specific and salivary gland-specific knock-down, fat body or salivary glands dissected from wandering third-instar larvae were used. RNA from homogenates were extracted as described above. Transcript levels were determined in RNA samples using qRT-PCR using the Invitrogen’s Superscript III Platinum SYBR Green One-Step qRT-PCR kit in. Primers were obtained from Integrated DNA Technologies (Coralville, IA). *Drosophila* ribosomal *protein 15a* was used as used as housekeeping. *Drosophila CG6364* (NCBI Accession #NM_142984) was detected using forward primer (5’TGTCCATCAGTCAGGACAGC 3’) and reverse primer (5’CTCCACTTTGTGGCCCTTTA 3’). The cycling condition used for the assay were: 48°C for 30 minutes, 95°C for 3 minutes, and then 45 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 60°C for 1 minute. *Drosophila San* (NCBI Accession # NM_080040) was detected using forward primer (5’ACCCGAACAATCAGGAACAG 3’) and reverse primer

(5'ACCCGAACAATCAGGAACAG 3'). The cycling condition used for the assay were: 48°C for 30 minutes, 95°C for 3 minutes, and then 45 cycles of 95°C for 15 seconds, 50°C for 30 seconds, and 60°C for 1 minute.

To calculate knock down efficiency serial 10-fold dilutions of RNA and corresponding Ct values were used to plot standard curves (mean of three experiments). Primer efficiency was calculated using the following equation (29):

$$\textbf{Efficiency} = 10^{(-1/\text{slope of standard curve})}.$$

Primer efficiency values were used to calculate the relative change in gene expression by the following equation (29):

$$\frac{(\text{Efficiency of gene interest})^{\text{Gene interest:}\Delta\text{Ct control} - \text{treated}}}{(\text{Efficiency of housekeeping gene})^{\text{Housekeeping gene:}\Delta\text{Ct control} - \text{treated}}}$$

Parental lines (*UAS* and *Gal4* constructs) and *yellow white* (*yw*) flies served as normal controls and were set at 100 % expression for the gene of interest. Relative level of gene expression in RNAi flies as compared to controls was calculated to estimate the knock down efficiency.

Infections.

Adult flies were used to assess the effect of gene knockdown on *E. chaffeensis* infections. Male and female flies were anesthetized on a CO_2 anesthesia pad (Genesee Scientific, San Diego, model# 59-119). Flies were injected in the thorax with 50.6 nl of sterile PBS-blue food dye solution (38% v/v) or *ehrlichia* resuspended in PBS-blue food dye solution (38% v/v).

Injections were made in the abdomen of the fly with pulled glass capillary needles using a Nanoject II (Drummond Scientific Company, Broomall, *PA*). Following injection, flies were maintained in clean bottles with molasses/yeast caps. Survival was monitored daily for 4 days.

Statistics.

Survival data were analyzed for significance using the log-rank test of Kaplan Meier plots using Prism Graphpad software (La Jolla, CA). Data are presented as the mean ± standard error (SE) of independent experiments. *P* values of <0.05 were considered highly significant. Bacterial numbers were analyzed by using the StatMost Statistical Package (Data XIOM, Los Angeles, CA, USA). Data are presented as the mean ± standard error (SD) of independent experiments. Statistical values were determined using the student's t-test (two-tailed, general).

Results

CG6364 and *San* are required for *in vivo E. chaffeensis* replication in adult *D. melanogaster*

E. chaffeensis is capable of infecting and completing its life cycle in S2 cells and adult flies (21, 22). By performing microarrays on S2 cells and screening mutant adult flies, previous work identified two host genes, *CG6364* (1) and *san* (2) that control the replication of *Ehrlichia in vivo* (10). In vertebrates, *E. chaffeensis* exhibits tropism for monocytes/macrophages (26). However, no clear requirements for cell or tissue tropism have been defined in ticks. To screen for arthropod tissues in which *E. chaffeensis* replicates, we employed whole-organism *in-vivo* RNAi to do tissue specific inactivation of *CG6364* (1) or *san* (2). We took advantage of a fly collection that consists of RNAi transgenes, and expressed through the binary *UAS-GAL4* system (5, 10). The availability of diverse set of *GAL4* fly lines allowed us to silence genes in a tissue specific manner to determine where *E. chaffeensis* replicate in arthropods (15).

To determine whether *CG6364* and *San* could be efficiently silenced using the *UAS-GAL4* system, we individually crossed transgenic flies carrying inverted repeats of the *CG6364* gene under the control of UAS to flies carrying *armadillo (arm)-Gal4* (ubiquitous *Gal4* insertions) to silence *CG6364* ubiquitously, in the F1 progeny. Using qRT-PCR, we confirmed transcript reduction of *CG6364* in the RNAi flies in comparison to wild-type, *yw* flies and parental lines (Table 2.1). After confirming an RNAi silencing effect on *CG6364,* flies were experimentally challenged with *E. chaffeensis. E. chaffeensis* bacteria were injected into the abdomen of wild-type, parental lines and *CG6364*-RNAi flies. We observed that there was approximately 48% death in wild-type and parental lines after 96 h in comparison to control flies injected with PBS (Figure 2.1; panel A) ($P<0.05$, log rank test). However, flies which had ubiquitous *CG6364*-RNAi displayed 85% survival which was significantly better than the wild-type and parental lines, (Figure 2.1; panel A), ($P<0.05$, log-rank test). When we assessed the ubiquitous *CG6364*-RNAi flies for infection we found that *E. chaffeensis* was cleared from the RNAi flies significantly better than wild-type and parental lines after 96 h (Figure 2.2; panel A) ($P<0.05$, t-test). These experiments confirmed that *UAS/GAL4* could be used to efficiently silence *CG6364* in adult flies and that *E. chaffeensis* infection in flies was dependent on a functional *CG6364* gene (20).

Previous studies from our group demonstrated that the gene *san* was necessary for *E. chaffeensis* infection in *D. melanogaster* (20). Therefore, we also silenced *san* in RNAi transgenic flies. We measured an average of 83 ± 5% reduction of *san* transcript levels in F1 RNAi flies (Table 2.1). When flies were challenged with *E. chaffensis san-RNAi* flies had

approximately 80% survival compared to *yw* and parental control flies (Figure 2.1; panel B) (*P<0.05*, log-rank test). Assessment of *E. chaffeensis* numbers in *san*-RNAi flies also showed 98% fewer bacteria in the F1 flies 96 h after experimental challenge (Figure 2.2; panel B) (*P<0.05*, t-test).

E. chaffeensis replication in adult *D. melanogaster*

Ubiquitous tissue silencing of *CG6364* and *san* impeded *E. chaffeensis* infection in adult *D. melanogaster*. Therefore, we screened an array of tissues for their ability to support bacterial replication. In vertebrates, *E. chaffeensis* exhibits a tropism for monocytes and macrophages (26). Therefore, we hypothesized that *E. chaffeensis* replicates in hemocytes, the insect equivalent to mammalian macrophages. To test this hypothesis, we silenced *CG6364* and *san* in the eyes, wings, hemocytes, fat body and salivary glands in a tissue-specific manner in adult flies. We used a eye-specific (*Gmr-Gal4*), wing-specific (*MS1096-Gal4*), hemocyte-specific (*Hml-Gal4*), fat body-specific (*YP1-Gal)* or salivary gland specific (*Fhk-Gal4*) *Gal4* constructs to generate F1 flies.

To confirm the tissue specific knock-down of targeted genes, we dissected whole-heads and wings to assess eye-specific and wing-specific adult knockdown. We observed an average of 88 ± 2% and 74 ± 15% reduction in transcript levels of *CG6364* and *san* respectively in whole heads of F1 transgenic flies generated using eye-specific *Gal4* construct in comparison to organs from wild-type and parental flies (Table 2.3). Similarly, we observed an average of 76 ± 9% and 82 ± 5% reduction in transcript levels of *CG6364* and *san* in the wings of F1 transgenic flies generated using wing-specific *Gal4* construct in comparison to experimental controls (Table

2.2). To analyze off-target RNAi silencing, we analyzed changes in transcript levels of targeted genes in fly-bodies without wings or whole-heads of F1 transgenic flies. These tissues showed negligible changes in transcript levels in comparison to wild-type and parental flies (Table 2.2, 2.3). This confirmed the tissue-specific targeting of genes.

Due to the difficulty associated with isolation of hemocytes from adult flies we used whole-flies to assess hemocyte-specific knockdown. RNA levels were reduced by an average of 79 ± 6% and 62 ± 4 % in hemocyte-specific *CG6364* and *san*-RNAi whole-flies, respectively, in comparison to experimental controls (Table 2.1). Fat bodies and salivary glands are difficult to dissect from adult flies. Since the RNAi effect is applicable at all stage of the *Drosophila* lifespan (10), we isolated the fat bodies and salivary glands from 3rd instar larvae to assess the silencing efficiency in the fat body and salivary glands of our RNAi flies. There was an average of 77 ± 8% and 66 ± 12% reduction in transcript levels in the dissected fat body from fat body-specific *CG6364* and *san*-RNAi third instar larvae, respectively, in comparison to dissected fat body from third instar control larvae (Table 2.4). Similarly, transcript levels in dissected salivary glands from salivary gland-specific *CG6364* and *san*-RNAi third instar larvae showed that RNA levels were reduced an average of 95 ± 2% and 90 ± 6%, respectively, in comparison to dissected salivary gland from control larvae (Table 2.5). However, negligible changes were observed in *CG6364* and *san* gene expression in RNA samples isolated from third instar larvae non salivary gland tissue (Table 2.5).

After confirming a tissue-specific RNAi silencing effect of *CG6364* and *san*, control and RNAi flies were experimentally challenged with *E. chaffeensis.* We observed that eye-specific and wing-specific knock-down of *CG6364* and *san* did not impact fly survival. RNAi flies were

as susceptible to *E. chaffeensis* infection similar to the wild-type and parental controls (Figure 2.3; panel A and panel B) (Figure 2.5; panel A and panel B) (*P>0.05*, log-rank test). Similarly, salivary-gland specific *CG6364* and *san*-RNAi flies were also as sensitive to *E. chaffeensis* infection as control flies (Figure 2.11; panel A and panel B) (*P>0.05*, log-rank test). However, hemocyte and fat body-specific *CG6364* and *san*-RNAi flies survived significantly better than the control flies after infection with *Ehrlichia* (Figure 2.7; panel A and panel B) (Figure 2.9; panel A and panel B) (*P<0.05*, log-rank test). Ninty six hours (96 h) after infection, 80 % of hemocyte-specific *CG6364*-RNAi and 85% of hemocyte-specific *San*-RNAi flies survived in comparison to an average of 50 % of *yw* and parental control flies. Similarly, after 96 h after infection 80% of fat body-specific *CG6364*-RNAi and 80% of fat body-specific specific *San*-RNAi flies survived in comparison to an average of 50% of *yw* and parental control flies.

There was no difference in bacterial replication in eye-specific, wing-specific and salivary gland-specific *CG6364* and *san* F1-RNAi flies comparison to control flies (Figure 2.4, panel A and panel B) (Figure 2.6; panel A and panel B) (Figure 2.12; panel A and panel B) (*P>0.05*, t-test). However, F1 RNAi flies with hemocyte-specific knock-down of *CG6364* or *san* had significantly fewer bacteria (Figure 2.8; panel A and panel B) (*P<0.05*, t-test). At 96 h time point, there were 98% and 95 % fewer bacteria in hemocyte-specific *CG6364* or *san*-RNAi flies respectively in comparison to controls (Figure 2.8; panel A and panel B). Similarly, there were 97% fewer bacteria in fat body-specific *CG6364* or *san*- F1 RNAi flies (Figure 2.10; panel A and panel B). These data confirm that *CG6364* and *san* are needed for *E. chaffeensis* replication and support the hypothesis that the bacteria replicate in the hemocytes or the fat body in adult *D.melanogaster*.

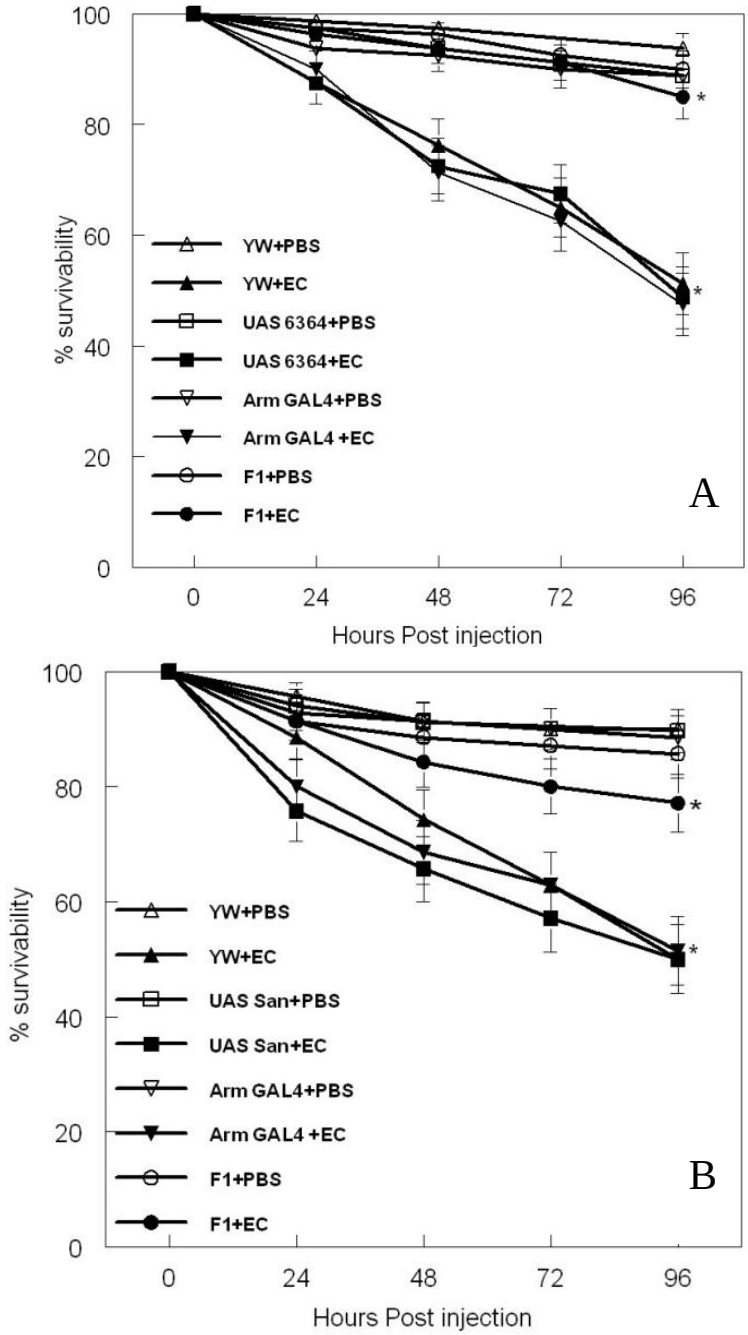

Figure 2.1 Impact of ubiquitous expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Ubiquitous knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

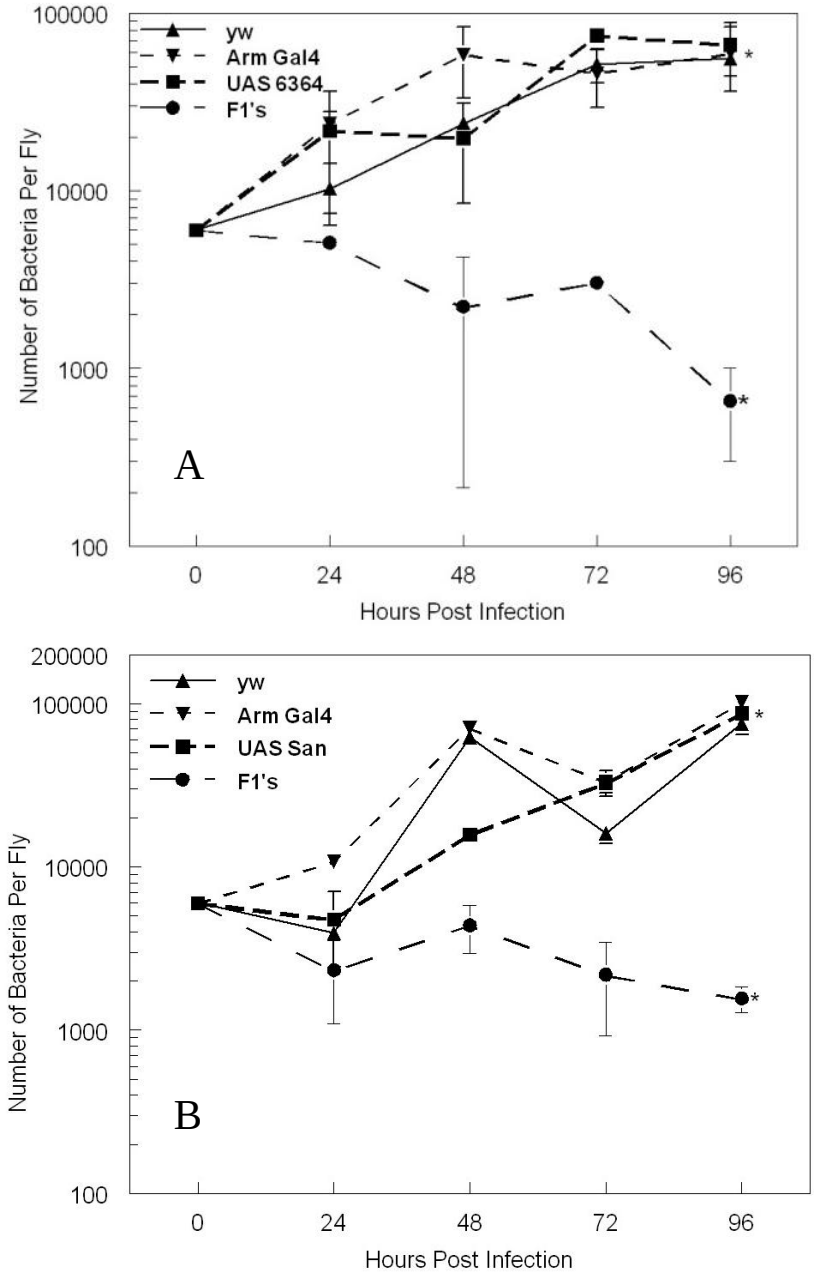

Figure 2.2 Impact of ubiquitous expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Ubiquitous knock-down of CG6364 and San was accomplished using UAS and Gal4 constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

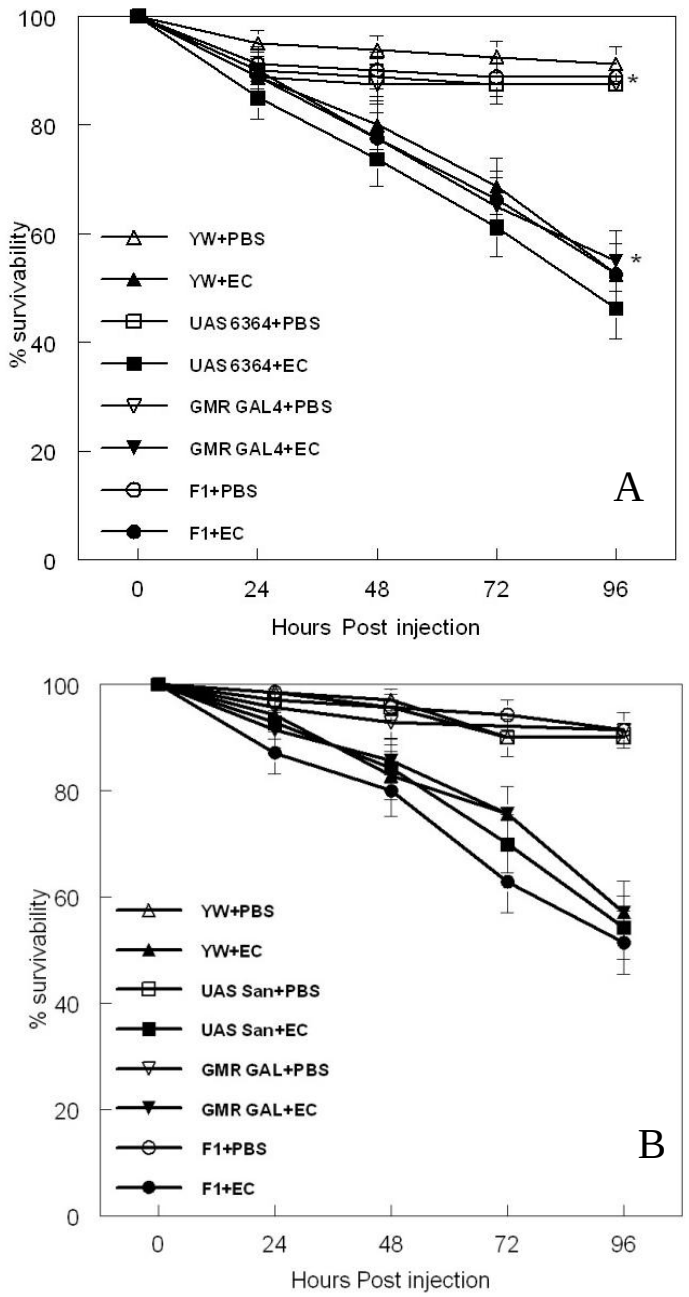

Figure 2.3 Impact of eye-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Eye-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

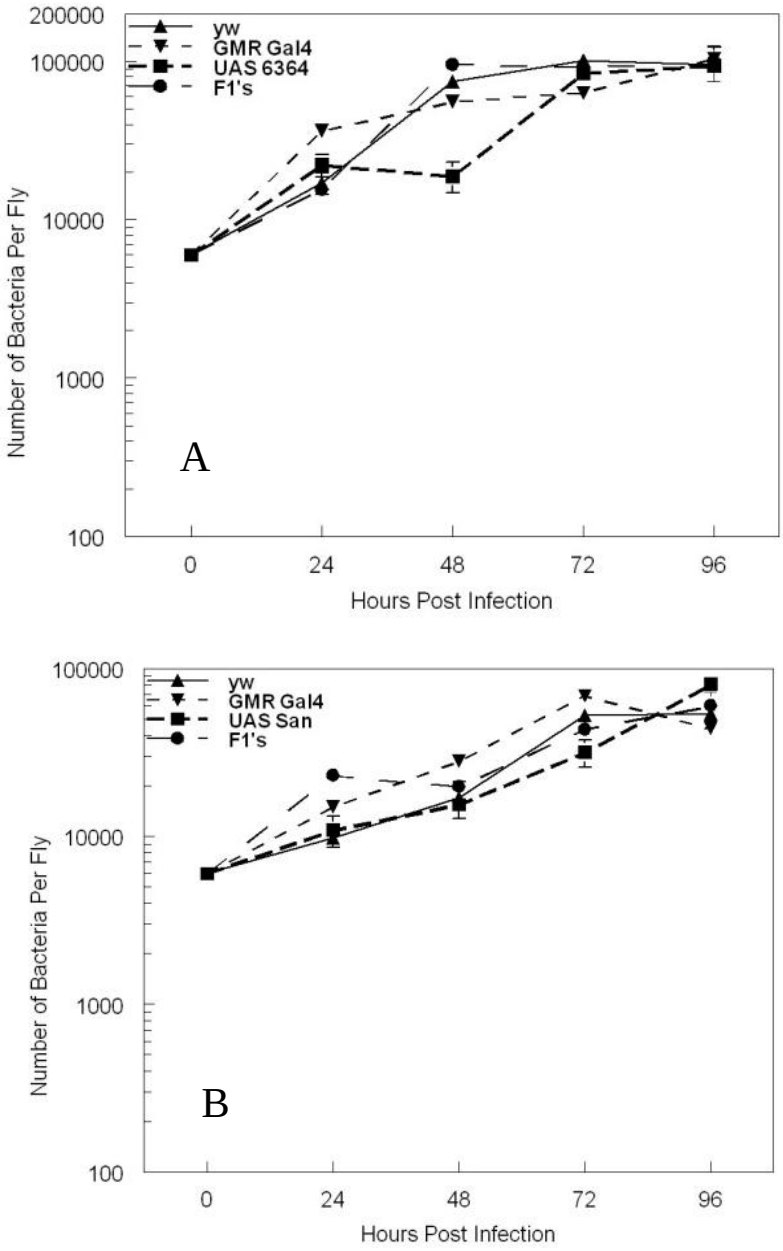

Figure 2.4 Impact of eye-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Eye-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$

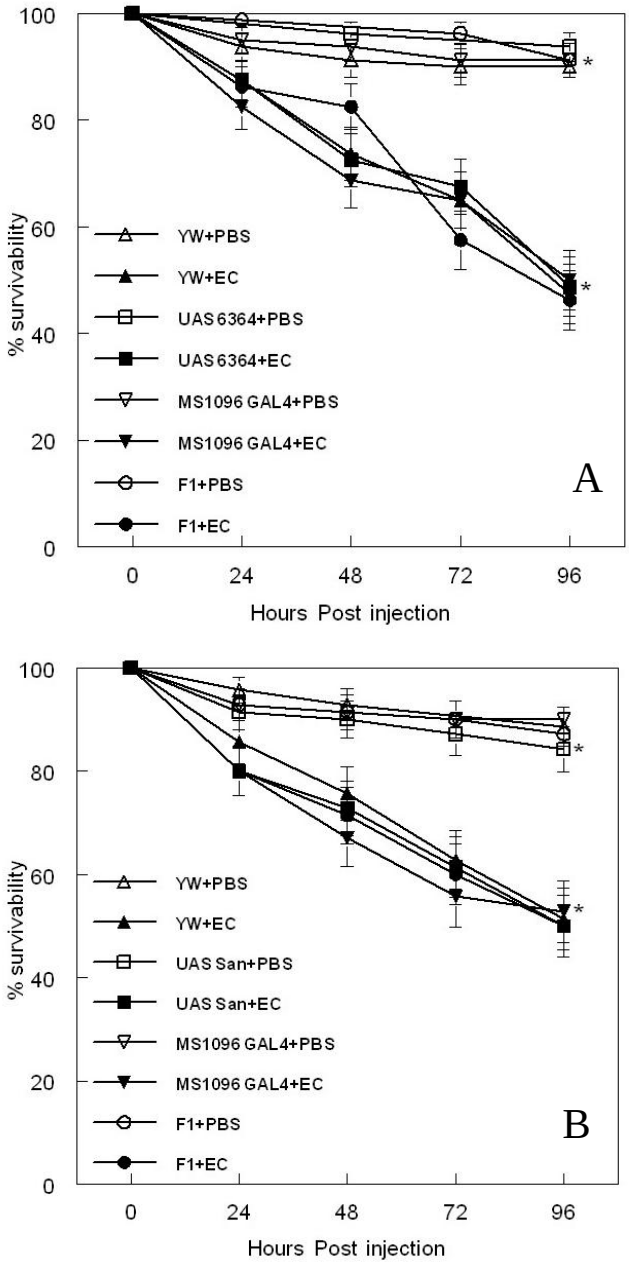

Figure 2.5 Impact of wing-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Wing-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

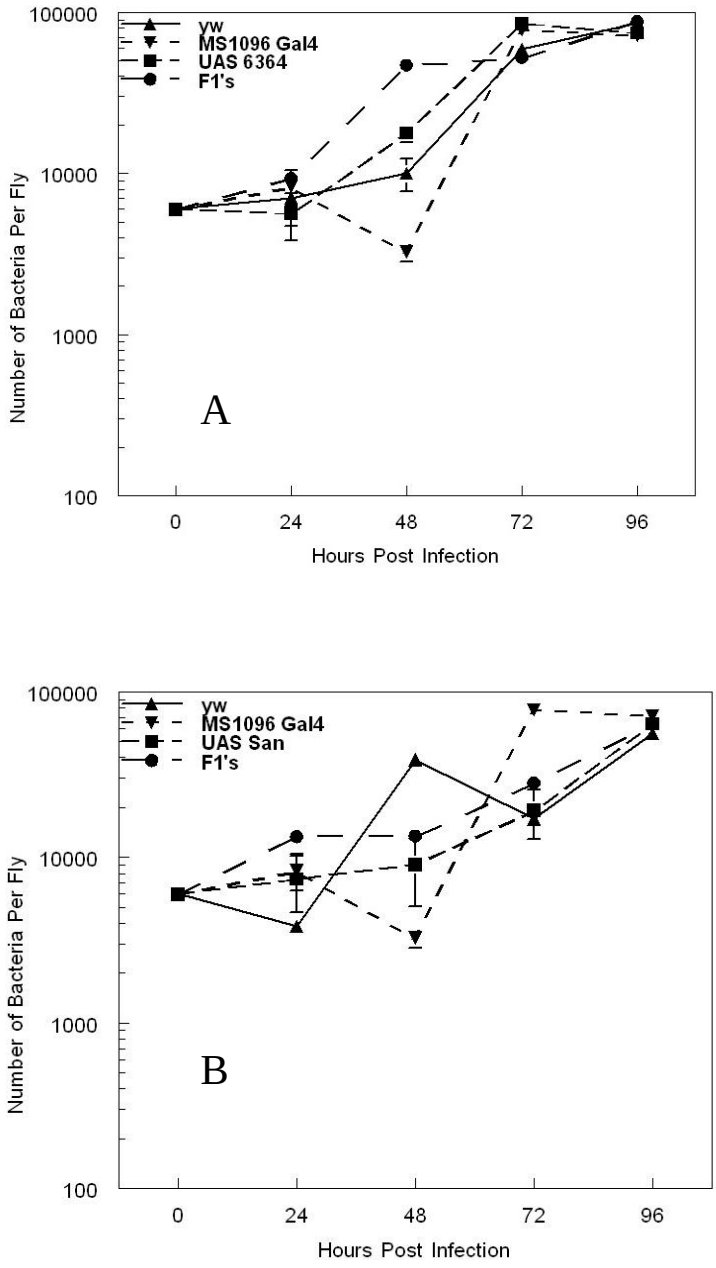

Figure 2.6 Impact of wing-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Wing-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

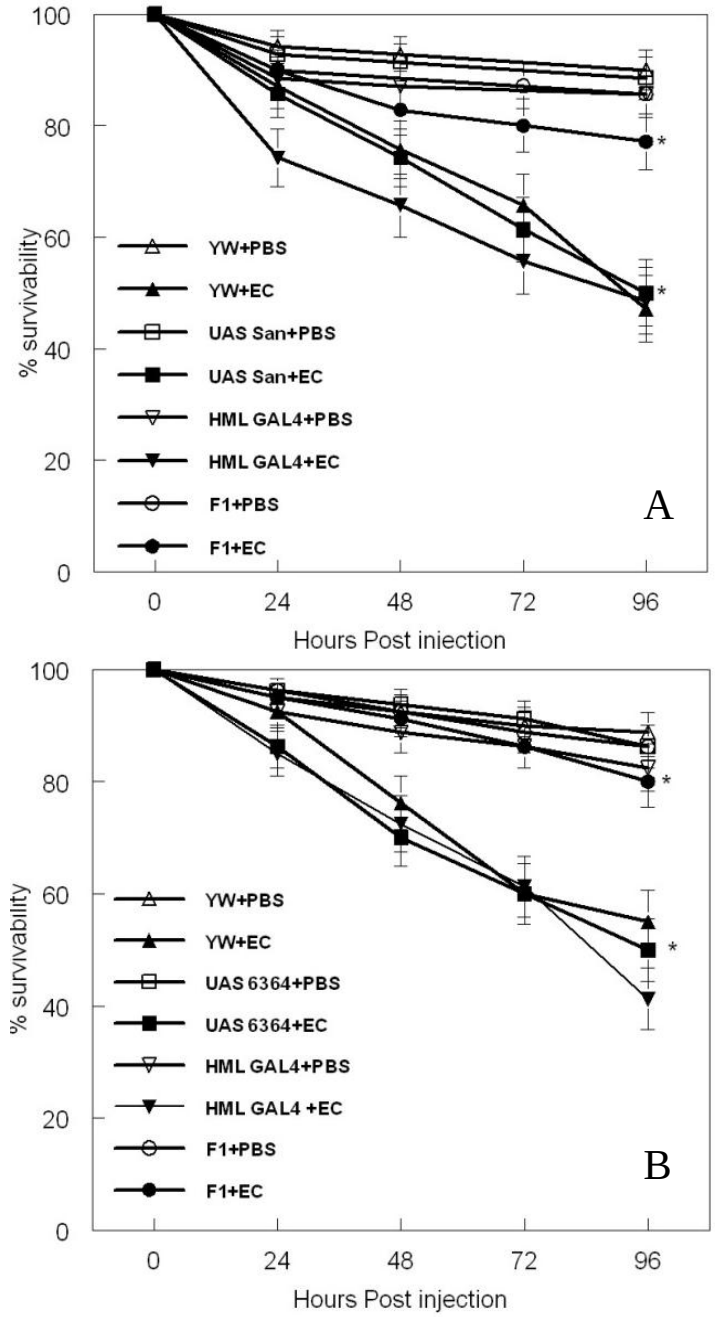

Figure 2.7 Impact of hemocyte-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Hemocyte-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

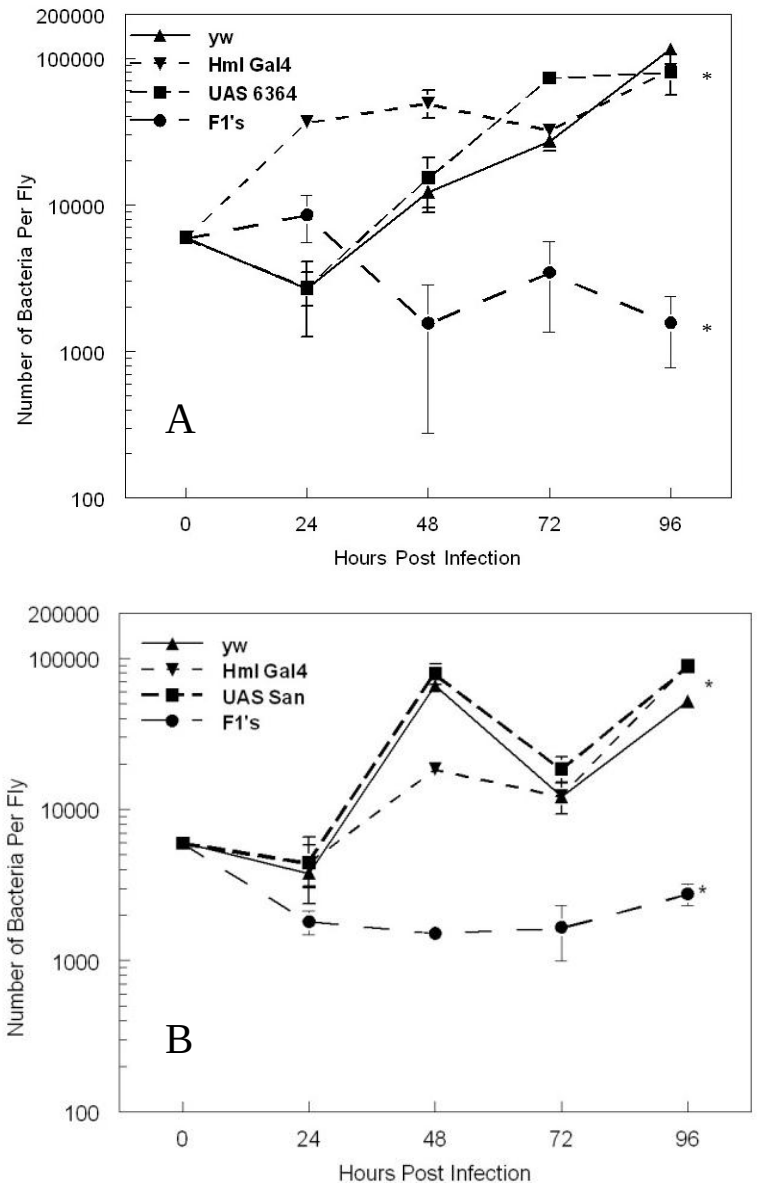

Figure 2.8 Impact of hemocyte-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Hemocyte-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

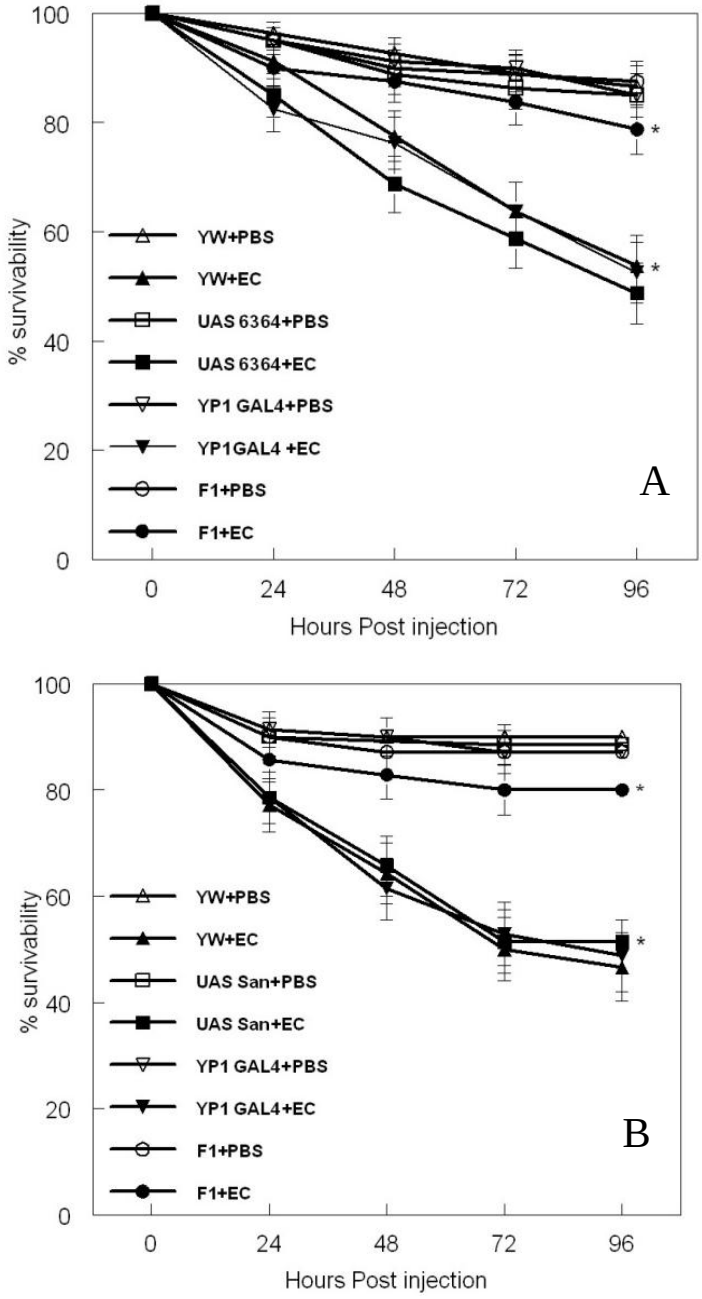

Figure 2.9 Impact of fat body-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Fat body-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

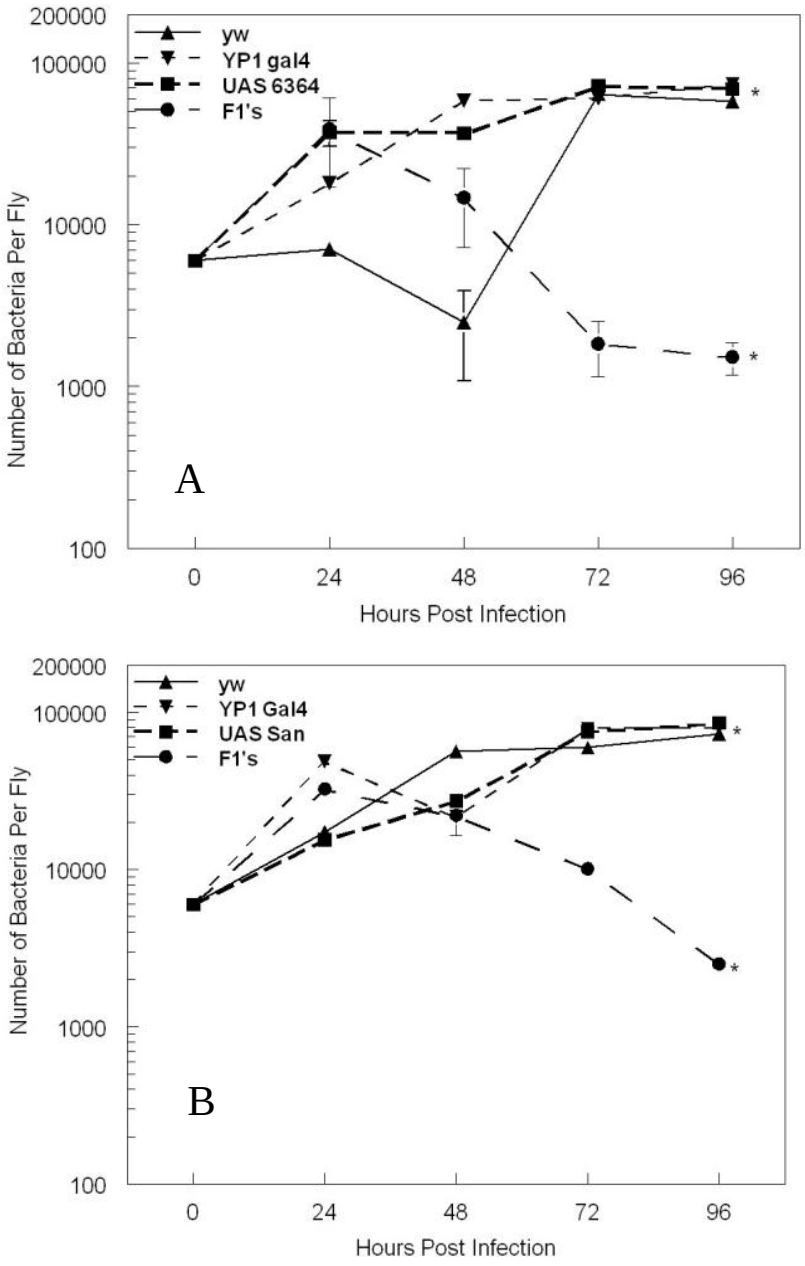

Figure 2.10 Impact of fat body-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Fat body-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

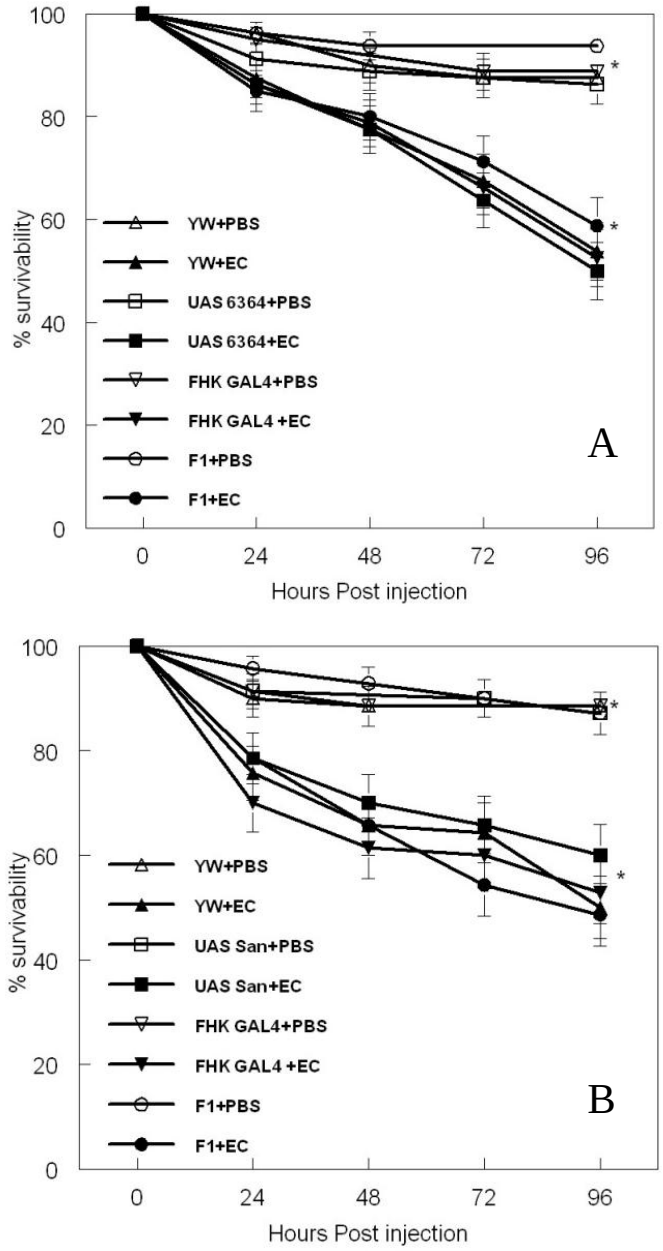

Figure 2.11 Impact of salivary gland-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on *E. chaffeensis* (EC) infection. Salivary gland-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Flies were injected with PBS or cell-free *E. chaffeensis*. Data presented represent the mean ± SEM of 3-4 independent experiments. 20 flies were injected per treatment group per experiment. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

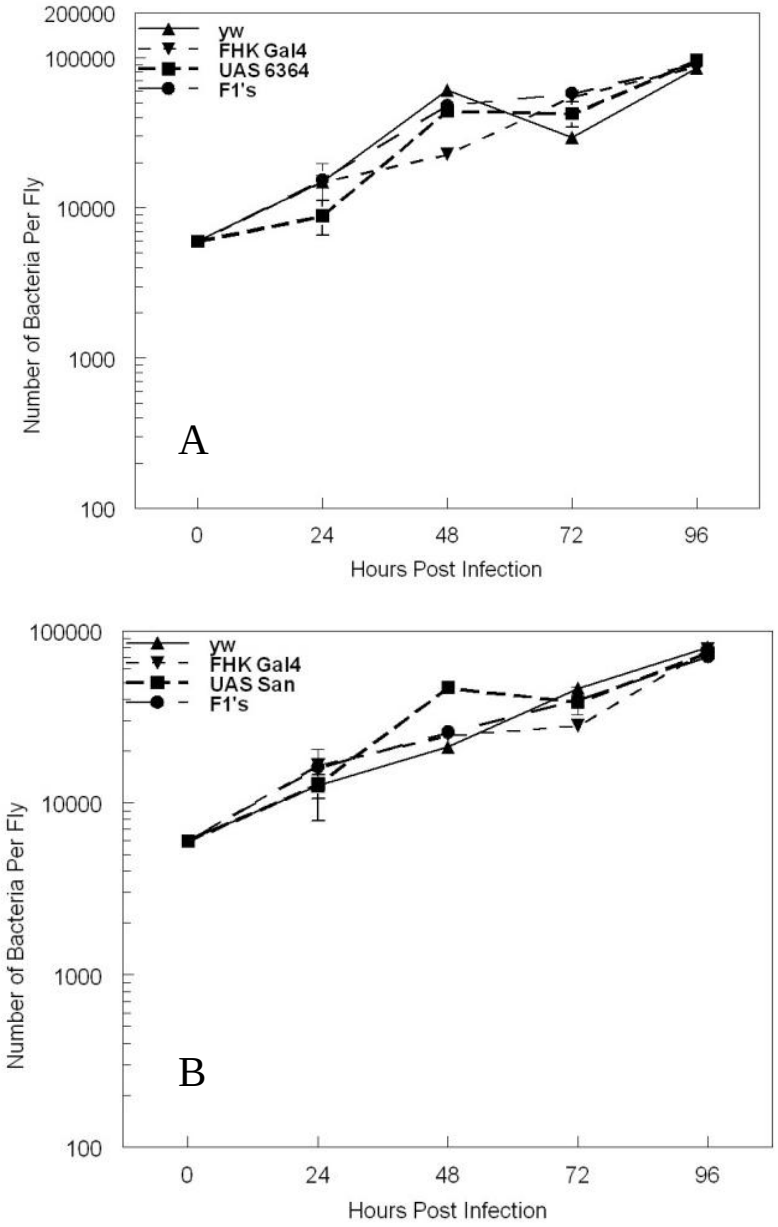

Figure 2.12 Impact of salivary gland-specific expression of *CG6364* (A) or *san* (B) hairpin RNA on bacterial clearance. Salivary gland-specific knock-down of *CG6364* and *San* was accomplished using *UAS* and *Gal4* constructs as described in materials and methods. Bacterial load was estimated by qRT-PCR for Ehrlichial 16S rRNA as described in the materials and methods. Data presented represent the mean ± SD of 2 independent experiments. Each point represents 4-5 flies per RNA preparation. The absence of error bars indicates an error smaller than the size of the marker. Statistical significance is represented by * ($P<0.05$)

Tissues in which the target gene was knocked down	**Target Gene**	**% knock-down efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls**	**% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (Parental line 1)**	**% knockdown efficiency of target gene in F1 progeny compared to *Gal4* construct (Parental line 2)**	**Average % knockdown efficiency of target gene in F1 progeny compared to control flies**
Ubiquitous (*arm-Gal4*)	*CG6364*	86 ± 4	90 ± 5	89 ± 9	88 ± 2
	San	86 ± 2	86 ±5	78 ± 4	83 ± 5
Hemocytes (*Hml-Gal4*)	*CG6364*	76 ± 8	85 ± 5	73 ± 3	79 ± 6
	San	61 ± 0	59 ± 2	66 ± 1	62 ± 4
Fat Body (*YP1-Gal4*)	*CG6364*	55 ± 1	26 ± 9	23 ± 1	35 ±18
	San	46 ± 2	28 ± 4	52 ± 3	42 ± 12

Table 2.1 Percent (%) knock-down efficiency of target gene in RNAi flies (F1's) in comparison to wild type and parental lines (*UAS* and *Gal4* constructs). RNA was isolated from whole-flies. Analysis of transcript level was done using qRT-PCR results as described in the materials and methods method. Data presented represent the mean ± SD of 2-3 independent experiments.

Tissues in which the target gene was knocked down	**Target Gene**	**% knock-down efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls**	**% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (Parental line 1)**	**% knockdown efficiency of target gene in F1 progeny compared to *Gal4* construct (Parental line 2)**	**Average % knockdown efficiency of target gene in F1 progeny compared to control flies**
Wings (*MSO196*-Gal4)	*CG6364*	81 ±2	66 ± 6	81 ± 7	76 ± 9
	San	77 ± 7	82 ± 22	86 ± 14	82 ± 5
Body (*MSO196*-Gal4)	*CG6364*	-26 ± 11	-8 ± 3	11 ± 2	-8 ± 19
	San	- 41	17	20	34 ± 3

Table 2.2 Comparison of knock-down efficiency in wing and whole body tissue wing-specific RNAi flies (F1's) in comparison to wild type and parental lines (*UAS* and *Gal4* constructs). RNA was isolated from wings or fly body (without wings). Analysis of transcript level was done using qRT-PCR results as described in the materials and methods method. Data presented represent the mean ± SD of 2-3 independent experiments.

Tissues in which the target gene was knocked down	**Target Gene**	**% knock-down efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls**	**% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (Parental line 1)**	**% knockdown efficiency of target gene in F1 progeny compared to *Gal4* construct (Parental line 2)**	**Average % knockdown efficiency of target gene in F1 progeny compared to control flies**
Whole heads (*Gmr Gal4*)	*CG6364*	86 ± 4	88 ± 5.	89 ± 9	88 ± 2
	San	57 ± 2	85 ±7	81 ± 7	74 ±15
Body (*Gmr Gal4*)	*CG6364*	23 ± 4	12 ± 3	8 ± 3	14 ± 8
	San	10 ± 0	25 ± 6	9 ± 3	15 ± 10

Table 2.3 Comparison of knock-down efficiency in whole heads and whole body tissue of eye-specific RNAi flies (F1's) in comparison to wild type and parental lines (*UAS* and *Gal4* constructs). RNA was isolated from whole heads or fly body (without theads). Analysis of transcript level was done using qRT-PCR results as described in the materials and methods method. Data presented represent the mean ± SD of 2-3 independent experiments.

Tissues in which the target gene was knocked down	**Target Gene**	**% knock-down efficiency of target gene in F1 progeny compared to wild-type (*yw*) controls**	**% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (Parental line 1)**	**% knockdown efficiency of target gene in F1 progeny compared to *Gal4* construct (Parental line 2)**	**Average % knockdown efficiency of target gene in F1 progeny compared to controls**
Fat body (*YP1-Gal4*)	*CG6364*	75	85	70	77 ± 8
	San	79	57	62	66 ± 12

Table 2.4 Comparison of knock-down efficiency in dissected fat body tissue of fat body-specific RNAi third instar larvae (F1's) in comparison to wild type and parental larvae (*UAS* and *Gal4* constructs). RNA was isolated from dissected fat body tissue**.** Analysis of transcript level was done using qRT-PCR as described in the materials and methods method. Data presented represent the mean ± SD of 1 independent experiment

Tissues in which the target gene was knocked down	**Target Gene**	**% knockdown efficiency of target gene in F1 flies compared to wild type (yw) controls**	**% knockdown efficiency of target gene in F1 progeny compared to *UAS* construct (Parental line 1)**	**% knockdown efficiency of target gene in F1 progeny compared to *Gal4* construct (Parental line 2)**	**Average % knockdown efficiency of target gene in F1 progeny compared to controls**
Salivary glands (*Fhk-Gal4*)	*CG6364*	92	97	96	95 ± 2
	San	93	96	82	90 ± 6
Body (*Fhk-Gal4*)	*CG6364*	16	32	36	28 ± 9
	San	18	22	30	23 ± 5

Table 2.5 Comparison of knock-down efficiency in dissected salivary gland tissue of salivary gland-specific RNAi third instar larvae (F1's) in comparison to wild type and parental larvae (*UAS* and *Gal4* constructs). RNA was isolated from dissected salivary gland tissue of whole larvae (without salivary gland)**.** Analysis of transcript level was done using qRT-PCR as described in the materials and methods method. Data presented represent the mean ± SD of 1 independent experiment

Discussion/Conclusion

The data presented in this thesis demonstrate that *E. chaffeensis* replicate in the hemocytes and fat body and fail to replicate in the eyes, wings or the salivary glands of adult *D. melanogaster*. *E. chaffeensis* are transmitted from ticks to their hosts through the bite of ticks (26). However, the tick system is not very well defined. Therefore, we have used a genetically tractable system and defined the replication sites of *E. chaffeensis* in arthropods. Although, fruit flies and ticks have different life-cycles the study provides insights to possible replication sites of *E. chaffeensis* in ticks.

E. chaffeensis exhibits tropism for macrophages/ monocytes (26). However, no clear tropism has been defined in ticks. Consequently, establishing the replication sites in which this bacterium replicates in arthropod is important for complete understanding of the pathogenesis of the disease. Hemocytes are the arthropod host equivalent of macrophages (19). The fat body is functionally equivalent to mammalian liver (19). Our data suggesting that *E. chaffeensis* replicates in the hemocytes is consistent with previous observation that *Rickettsia rickettsii* invade hemocytes in *Ixodid* ticks (35).

Previously, our group found that *E. chaffeensis* is capable of replicating in hemocyte-like phagocyic S2 cells (21). S2 cells have hemocyte-like properties and express a variety of hemocyte-markers such as Hemolectin *(Hml)* (8), Hemese *(He)* (18), *Drosophila* scavenger receptor-CI (*dSR-CI*) (27) and croquemort, a member of the CD36 super family (16). However,

the similarity of these cultured cells to hemocytes is an open question as evidences suggest that these cells have combined properties of plasmatocytes and crystal cells (9). Therefore, the *in vivo* data confirms that *E. chaffeensis* replicate in the hemocytes of adult *D. melanogaster*. This is consistent with the behavior of other intracellular pathogens are capable of avoiding the phagocytic pathway and replicate within *Drosophila* hemocytes. These pathogens include *Salmonella typhimurium* (6), *Listeria monocytogenes* (24), *Mycobacterium marinum* (12), *Legionella pneumophila* (13), and *Francisella tularensis* (38).

E. chaffeensis did not replicate in the salivary gland of adult *D. melanogaster*. These findings were intriguing since *A. phagocytophilum* has been detected in the salivary glands experimentally infected ticks (30). In our experiments, *Drosophila* were infected in the abdomen with *E. chaffeensis*. This is not similar to the natural route of infection in a tick. Ticks acquire the infection by a blood meal (26). Thus, the dissemination of *E. chaffeensis* to different tissues may vary due to differences in route of infection. It may also be possible that salivary gland physiology may vary among arthropods depending on the life-cycle, which may account for inability of *E. chaffeensis* to grow in the salivary-glands of adult *D. melanogaster.* Moreover, *Drosophila* live on yeast growing on decaying fruit/food. One may assume that they are better adapted to fight potential pathogenic micro-organisms in the salivary glands.

Interestingly, we observed 97% fewer bacteria post 96 h of infection, in fat body-specific *CG6364* or *san*- F1 RNAi flies compared to wild-type and parental controls. One might expect that because there is tissue-specific knock-down that some residual bacterial replication would occur. Because this was not seen, the data suggest that *E. chaffeensis* were also incapable of

replicating within the hemocytes of the fat body-specific *CG6364* and *san*-RNAi flies. This observation suggests that there might be coordination between the hemocytes and the fat-body. In fact, several observations indicate that hemocytes can signal to the fat body to regulate the humoral immune response (3, 7, 11, 33). Thus, signaling between the hemocytes and other immunocompetent tissue such as the fat body may play a critical role in coordinating the cellular and humoral immune response to ensure efficient defense of the organism.

The results in the thesis support that *Ehrlichia* replicate within the hemocytes of adult *D. melanogaster*. Previously, our group also demonstrated that *E. chaffeensis* is capable of replicating in hemocyte-like phagocytic S2 cells (21). These data support that the tissue-tropism of *E. chaffeensis* to phagocytic cells arose approximately 600 million years ago, since that is when mammals and dipterans last shared a common ancestor (17, 28). It is not clear, however, whether this tropism will also be seen in *A. americanum*, the vector for *E. chaffeensis*. Ticks and dipterans last shared a common ancestor approximately 500 million years ago (37). Therefore, one would expect the tropism in hemocytes to be similar in ticks. However, genetic drift could have occurred in the *Ecdysozoa*, *Arthropoda* or later in the *Chelicerata* (17). Therefore, this hypothesis needs to be confirmed with experimental evidence.

The observation that *E. chaffeensis* is unable to replicate and cause infections in flies that had suppressed in *CG6364* or *san* is consistent with previous findings using mutant flies. *CG6364* is thought to be uridine/cytidine kinase (1). Luce-Fedrow et al. found that supplementation of cytosine to S2 cell culture enhanced *E. chaffeensis* replication (20). The investigators suggested that since cytidine is the least abundant nucleoside in cells, its use by *Ehrlichia* may be stressful on the host cell process which would make conditions amenable for *E.*

chaffeensis growth (20). The human homolog of *CG6364* is *uridine cytidine kinase 2* (*UCK2*) (1). The molecular function of *san* is defined as fatty acid binding/mitotic sister chromatid binding (2) and its human homolog is *N(alpha)-acetyltransferase 50, NatE* catalytic subunit for *Drosophila san* (2). This can open avenues to finding homolog genes in humans that is a prerequisite for the bacteria to replicate.

In conclusion, we have demonstrated that *E. chaffensis* replicates in the hemocytes and fat body of adult *D. melanogaster*. The study has successfully identified the intracellular niches that *E. chaffensis* replicate within arthopods. This provides insights to replication sites of *E. chaffeensis* in ticks. Using an RNAi approach, we also confirmed the requirement of *CG6364* and *san* for *in vivo E. chaffeensis* replication. Finding of conserved factors in humans from this investigation has a potential in translational research, immunodiagnostics, drug designing and RNAi therapeutics.

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